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14. ABSTRACT

The goals of the second year/phase of the DOD funded research, PC073499, have been accomplished. We produced two human versions of the OX40 ligand:trimer:Ig protein. The two protein constructs have different human trimer domains (TRAF2 and Matrillin-4), which confer differing folding patterns within the two-dimension structure of the protein. These two fully human OX40L:Ig fusion proteins were administered to non-human primates to test for in vivo activity. The OX40L:Ig fusion proteins showed potent biologic activity in vivo, but it was clear that the one with the TRAF2 trimer domain was superior. Therefore we are moving forward with OX40L:Ig fusion protein with the TRAF2 trimer domain with the ultimate hope of taking it to clinical trials in patients with prostate cancer.

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Laboratory-Clinical Transition Award: Stage I

PC073499 – Production and Characterization of a Novel OX40 Ligand for Clinical Use.
PI -Andrew Weinberg, PhD

INTRODUCTION: Cancer cells have evolved to evade immune-mediated destruction through several documented mechanisms. Our group has developed a technique to enhance immune function in tumor-bearing hosts through the use of OX40 agonists, which can lead to regression of tumors of various histologies, including prostate cancer [1]. In particular, we have produced a human OX40 agonist, termed OX40L:ILZ:Ig that has potent biologic function in vitro and is produced in large quantities by tissue culture cells. The ILZ portion of the chimeric protein was initially a trimerization domain obtained from a yeast sequence. Our goal in the first year of funding was to fully humanize this molecule by incorporating human trimerization domains to replace the yeast sequence, thus lowering the probability of immune-mediated recognition of this recombinant protein by treated cancer patients. This goal was accomplished with two human trimerization domains from the TRAF2 and Matrilin-4 proteins. Now that a fully functional human OX40 ligand protein was developed we proceeded with production and purification of the protein so that it could be tested for in vivo biologic activity in non-human primates. In the second year of funding we found that the biologic activity of the fully human OX40L:Ig protein was as potent as another OX40 agonist Ab that we currently have administered to patients. In the third year we will produce a cell line that is GMP compliant and this cell line will be used to produce this protein for toxicity studies and clinical trials. The ultimate goal of the research is to produce clinical grade human OX40L:ILZ:Ig to test in clinical trials for patients suffering from prostate cancer.

BODY: The first year of funding was spent on perfecting the soluble OX40 ligand molecule. In particular, we proposed to swap the yeast ILZ domain with known human trimerization domains. After modeling the different known human trimerization domains we made a decision to test two types: 1) a non-covalent trimerization domain (from TRAF2 sequence) and 2) a disulfide linked covalent trimerization domain (from matrilin-4 sequence). TRAF2 is an adapter protein that directly associates with the OX40 cytoplasmic tail and ultimately is involved with transmitting a downstream signal to the nucleus. It has been shown that OX40 and OX40 ligand form a trimer at the cell surface and the associated TRAF protein(s) from a trimer within the cytoplasm essentially forming a stacked three protein trimer complex. Hence we hypothesized that the TRAF2 trimer domain might make a perfect spatial fit between the OX40L extracellular domain and the Ig tail. There are only a few of the human trimerization domains that form natural disulfide bonds and the matrilin-4 protein is one of them. We reasoned that a covalently bonded trimerization domain might offer greater stability in vivo and hence might have increased biologic activity when compared to a non-covalent trimer domain. Therefore we produced both OX40L constructs and expressed them in 293 cells as secreted proteins. Protein G column chromotography was used to purify the proteins to >95% homogeneity and we compared their size by native gel electrophoresis. Prior to these experiments we published [2] that our initial recombinant protein, OX40L:ILZ:Ig, containing the yeast trimerization domain folded predominantly as a hexamer as determined by column chromatography. It was clear that the TRAF2 and matrilin-4 trimerization domains gave similar protein products (3 bands) but the matrilin-4

protein was less homogenous compared to the TRAF2 construct. The matrilin-4 domain clearly had more of a higher and lower MW, which may or may not correspond to increased biologic activity. The initial bioassay results showed that both protein constructs have potent biologic activity in T cell stimulation assays. While it is hard to pick a clear cut "winner" based on our in vitro assays, both of these proteins were tested side by side in a non-human primate pilot study performed in year 2.

Our initial Statement of Work did not include the non-human primate pilot study discussed above, however in November 2009 we sent a revised statement of work that included the pilot study and shortly thereafter it was approved. These fully human OX40L proteins needed to be tested in vivo prior to moving forward with making material for toxicity studies and a phase I clinical trial. The human OX40L:Ig proteins do not bind to mouse or rat or other species and therefore had to be tested in non-human primates. The pilot study tested the fully human OX40L:Ig agonists and compared them to the murine anti-human OX40 Ab that has just completed phase I testing in cancer patients. Mouse Ig was used as a negative control in these experiments (see Fig 1 for experimental design). Based on our clinical trial experience and previous OX40 agonist monkey toxicity data [3] we picked a dose of 1 mg/kg for all agents and this was delivered to four monkeys per group. In Fig 1 we show a schematic of the experiment, with the OX40-specific agents delivered on days 0, 2, and 4. The monkeys were injected with tetanus prior to infusion and pharmacokinetic points were taken pre- and 30 minutes post-infusion for all three infusions. We drew blood at multiple time points after infusion so that we could ascertain changes in T cell proliferation through the Ki-67 marker [4] and also assess whether these new OX40 agonists could increase an Ab response to tetanus.

We first assessed the pharmacokinetics of all three OX40 agonists, shown in Fig 2. Prior to the monkey study we knew that the OX40L:Ig constructs were shorter lived (from previous mouse experiments) and we found the same was true when they were injected into monkeys. The serum levels of both the TF2 and MT4 constructs increased 30 minutes after infusion and then two days later were barely detectable. This was true for all three infusions. We also found that the peak level of OX40L in the serum was about 5-fold less than the peak OX40 mAb levels. It was also clear that the OX40 mAb lasted for a greater duration, as detectable levels were found 3 days after the last infusion.

Despite the short-lived pharmacokinetics, the OX40L:Ig TF2 fusion protein produced similar levels of biologic activity in the monkeys when compared to the CD134 mAb, as assessed by Ki-67 expression in peripheral blood T cells and serum tetanus Ab titers (Figs 2-5). Figure 2 shows a representative monkey from groups A, C, and D depicting raw flow cytometry-based data, which were gated on $CD4^+/CD95^+$ T cells and assessing Ki-67 vs CD28 over time. Similar to what was observed with our mouse anti-human OX40 agonist in the phase I clinical trial there was an increase in Ki-67 staining starting 7 days after the initial OX40 agonist infusion. This increase peaked 14 days after the initial infusion (Figs 2 & 3) and declined thereafter. We performed statistical evaluation at the day 14 time-point and found that all three test groups were significantly increased compared to the negative control (Fig 3B). The majority of the ex vivo Ki-67 examination showed that the OX40L:Ig TF2 protein out performed the MT4 construct in vivo. The TF2 OX40L:Ig construct also showed greater molecular homogeneity when compared to the MT4 construct therefore we have picked the OX40L:Ig TF2 protein as our "lead" agent to move forward with. We next analyzed Ki-67 expression in the central memory CD4 T cell population (as defined by $CD28^+/CCR5^-$ [5]) and found that the OX40L TF2 protein showed a greater increase compared to

the MT4 and the anti-OX40 Ab at days 7 and 11 post-infusion (Fig 3B). This data suggested that the OX40L:Ig protein may have immune stimulatory properties that are indeed different/superior than the mAb in vivo and it has been postulated that increases in central memory T cell populations is a good indicator of tumor rejection in vivo [6]. We next examined increases in CD8 memory T cell Ki-67 expression. The anti-OX40 Ab showed the greatest increase in CD8 T cell proliferation (Fig 4A), however when the statistics were assessed on day 14 only the OX40L:Ig TF2 protein and the CD134 mAb showed a significant increase in CD8 memory T cell Ki-67 expression when compared to the mIgG control (Fig 4B). Finally, we assessed a tetanus-specific Ab response in the four groups of treated monkeys. We previously found that monkeys treated with an OX40 Ab showed increased titers to immunizing Ags [3]. We found that monkeys treated with either anti-OX40 or either OX40L:Ig fusion protein showed increased serum Abs to tetanus. Figure 5 shows tetanus Ab titers for a representative monkey in the OX40L:Ig TF2, CD134 mab, and mouse IgG groups (A, B, and C respectively). As summarized in Fig 5D the average tetanus serum Ab titers were increased approximately 5-fold in both the OX40L:Ig TF2 and CD134 mab groups when compared to the mouse IgG group. Thus, this data confirms that the OX40L:Ig TF2 protein had similar levels of biologic activity when compared to the anti-OX40 Ab and in some cases greater activity (CD4 central memory proliferation).

Based on the data shown above we have now picked the OX40L:Ig TF2 as our lead agent and will be pursuing its production in the coming months. In order to produce large quantities for clinical trials it is imperative that we pursue cell line development that will ultimately allow for increased production of this protein. This is the next step that will pursue with our lead agent, however this was not listed within our initial statement of work. Therefore in the coming weeks we will submitting a revised statement of work to include this important piece of work that will ultimately get us closer to administering this OX40 agonist to patients with prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS:

- We produced two novel forms of the human OX40 ligand:trimer:Ig protein that have completely human sequences.
- These proteins were injected into non-human primates and assessed for biologic activity.
- It is clear that the TRAF2 trimer and the Matrilin-4 trimer domains were able to produce biologically active OX40 ligand proteins in vivo, but the TRAF2 construct was superior.
- When the OX40L:Ig proteins were tested side by side with our mouse anti-OX40 antibody there was similar amount of in vivo activity, although the OX40L:Ig fusion protein did show enhanced activity in stimulating proliferation of central memory CD4 T cells.
- The results from the non-human primate pilot study were successful and have generated much enthusiasm for moving the OX40L:Ig TRAF2 protein forward for future clinical trials.

REPORTABLE OUTCOMES:

We have yet to publish these results as they somewhat confirmed what we already knew with OX40 agonists in the context of stimulating the murine immune system. However, there were some interesting caveats like the enhanced proliferation of central memory CD4 T cells that have not been previously reported. In collaboration with the Oregon Primate Center we are performing some follow-up studies, where we could incorporate the new findings with the ones presented in this report for a novel manuscript.

CONCLUSION: In summary, we have completed the second year/phase of the research, which was to complete production and purification the fully human OX40 ligand:trimer:Ig protein and test them in vivo. We were able to generate sufficient quantities of both proteins and purified them to greater than 95% homogeneity. We next tested these immune stimulatory proteins versus our mouse anti-human OX40 Ab that has already been tested in the clinic. The OX40L:Ig fusion proteins showed similar immune stimulatory properties as the mouse monoclonal OX40 Ab. It was clear that the OX40L:Ig fusion protein with the TRAF2 trimer domain had greater activity in vivo when compared to the MT4 trimer domain. Therefore we are going forward with the clinical development of the OX40L:Ig TRAF2 trimer protein.

In our phase I clinical trial with the mouse OX40 agonist Ab administered to stage IV cancer patients we have observed some tumor shrinkage in 7/30 patients, however we did not observe a complete response. Our hope is that the fully human OX40 agonists described herein will have more potent biologic activity because it could be administered several times unlike the mouse anti-human OX40 Ab. Ultimately, upon completion of this project we will have important preclinical information that will help in garnering FDA approval of a fully human OX40 agonist to treat prostate cancer patients.

FIGURES:

A

Group	Number of animals	Sex	agent
A	4	F	mouse IgG1
B	4	F	Fc:MT4:OX40L
C	4	F	Fc:TF2:OX40L
D	4	F	mouse anti-human OX40 (CD134 mab)

B

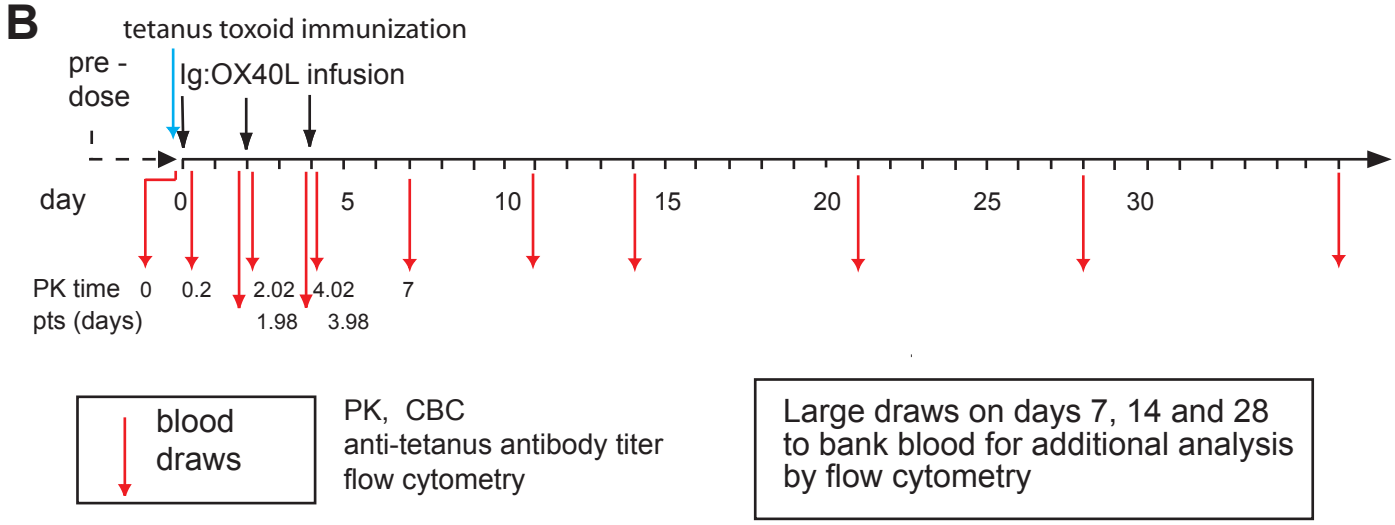

Figure 1. Schematic for testing the activity of recombinant OX40L in non-human primates. A. Schematic of treatment groups. Mouse IgG1 (MOPC21) was obtained from BioXpress. Fully human recombinant OX40L fusion protein containing the coiled-coil domain from either matrilin-4 (FcMT4OX40L) or from Traf 2 (FcTF2OX40L) were obtained by expression of these constructs in HEK 293 cells in a hollow-fiber bioreactor (FiberCell). Mouse anti-human OX40 was the same monoclonal antibody used in our phase I clinical trial. All reagents were prepared at 2.0 mg/ml in phosphate buffered saline. **B.** Rhesus monkeys were treated with OX40 agonists or controls on days 0, 2 and 4 by IV infusion at a dose of 1mg/kg. Blood samples were taken at the times indicated. For the analysis of pharmacokinetics, samples were taken 30 minutes before and 30 minutes after each infusion. Whole blood was used for phenotypic analysis by flow cytometry and for CBC. Plasma was prepared for PK analysis and for measuring antibody titers to tetanus toxoid.

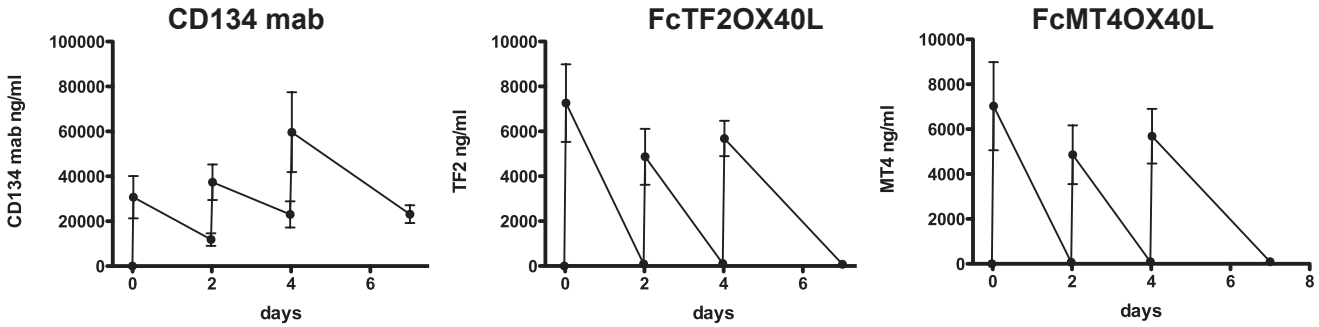

Figure 2. Pharmacokinetics of recombinant OX40L (FcTF2OX40L and Fc MT4OX40L) compared to OX40 agonist mouse monoclonal antibody (CD134 mab) in Rhesus monkeys. Blood samples were obtained according the scheme in figure 15, (30 minutes before and after the I.V. infusions on days 0, 2 and 4 and then on day 7). Values for individual animals were combined to obtain the group means at each time pont. The concentrations of circulating FcTF2OX40L and CD134 mab were determined by ELISA. For FcTF2OX40L: anti-rat IgG (Fcγ) capture antibody - rat anti-human OX40L - serum samples - anti-human IgG Fcγ conjugated to HRP. For CD134 mab: anti-mouse IgG Fcγ - serum samples - anti-mouse IgG conjugated to HRP.

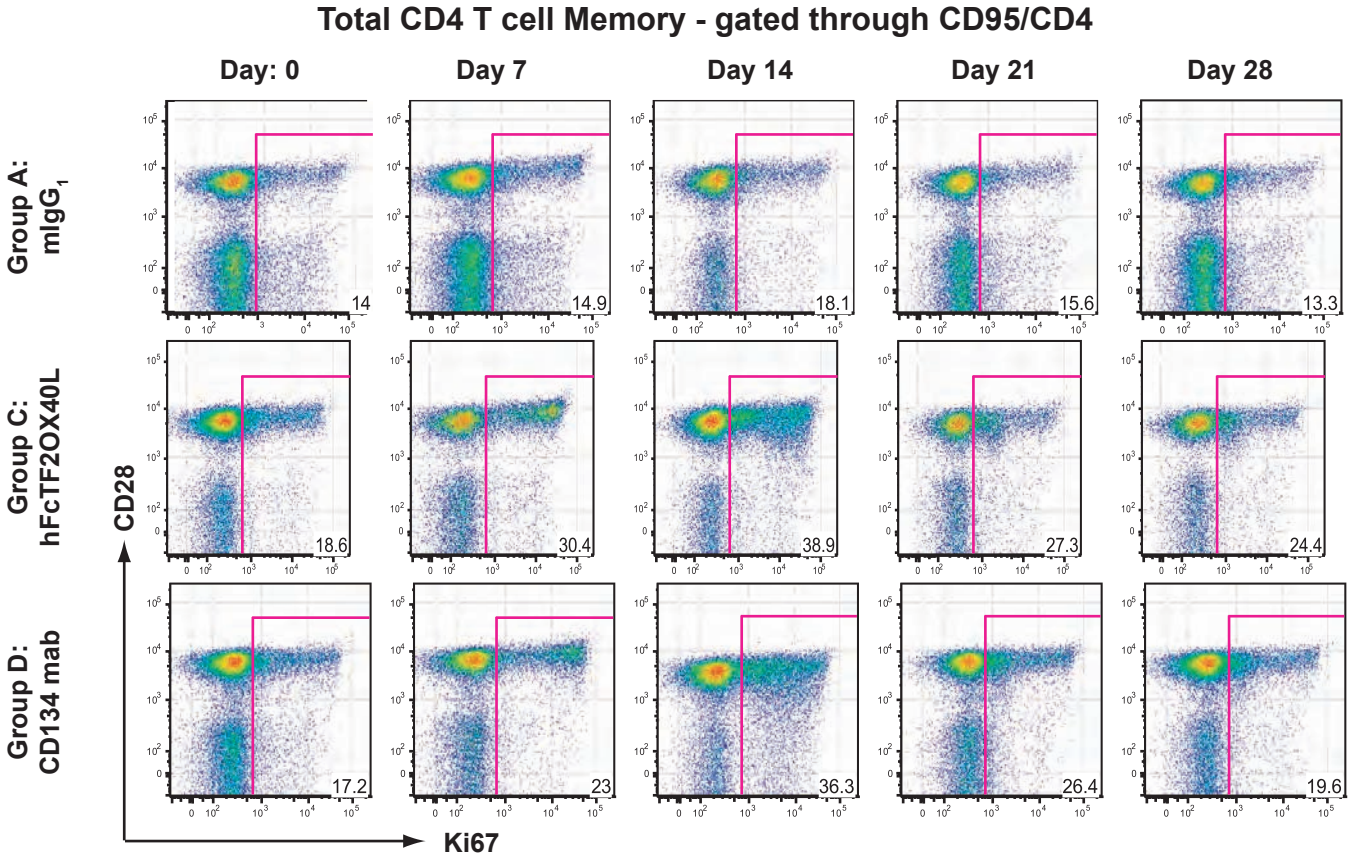

Figure 3. Proliferative response of CD4 T cell memory populations to OX40 engagement using Ki67 as a marker for T cell proliferation. Whole blood assays to assess T cell phenotypes by flow cytometry were performed at the times indicated. The lymphcytes were gated through CD3, CD4, and CD95. The data plots show CD28 vs Ki67 are shown. The number in the bottom right corner is the percentage of Ki67 positive cells, where an isotype control antibody was used to discriminate positive from negative events.

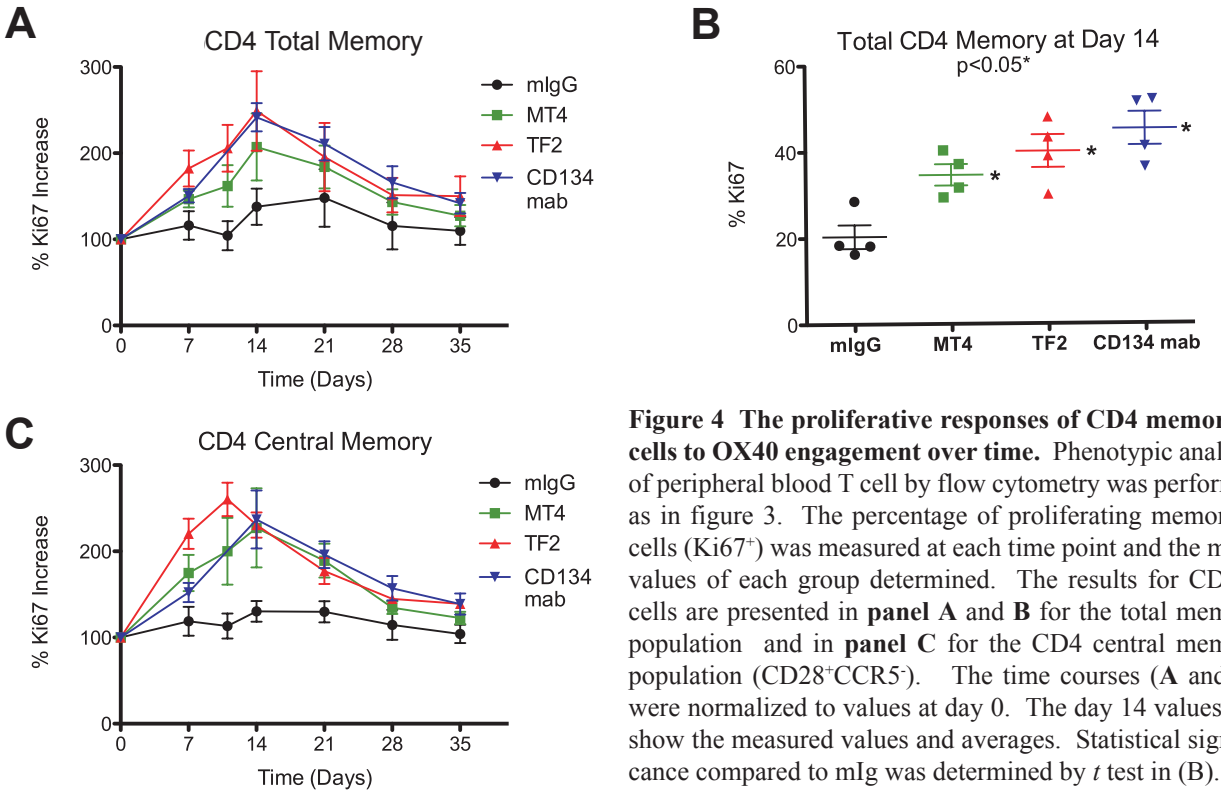

Figure 4 The proliferative responses of CD4 memory T cells to OX40 engagement over time. Phenotypic analysis of peripheral blood T cell by flow cytometry was performed as in figure 3. The percentage of proliferating memory T cells ($Ki67^+$) was measured at each time point and the mean values of each group determined. The results for CD4 T cells are presented in **panel A** and **B** for the total memory population and in **panel C** for the CD4 central memory population ($CD28^+CCR5^-$). The time courses (**A** and **C**) were normalized to values at day 0. The day 14 values (**B**) show the measured values and averages. Statistical significance compared to mIg was determined by *t* test in (B).

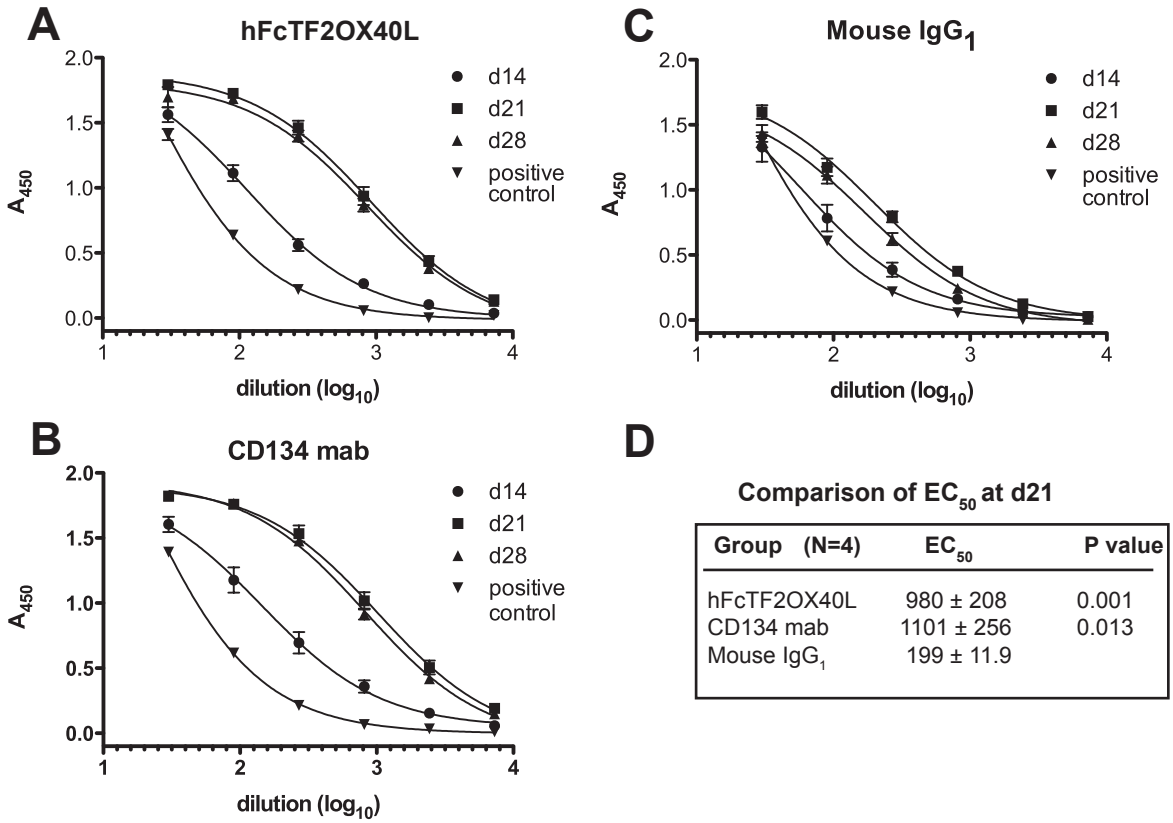

Figure 5. The effect of OX40 engagement on anti-tetanus toxoid antibody titers after vaccination. Plasma was prepared from blood samples taken at days 14, 21 and 28. Anti-tetanus titers were measured by ELISA using an HRP-conjugated secondary antibody that recognizes all classes of monkey antibodies (Rockland). The data for **(A)** OX40L:Ig, **(B)** CD134 mab, and **(C)** mouse IgG are the average curve fit to the data from all four monkey in each group. The titration data was fitted to a sigmoid dose response curve by the least squared method. For statistical comparison **(D)**, the EC_{50} for individual monkeys in each group was determined from the least squares fit. All four monkeys per group were averaged and these values evaluated by t-test for significance vs the mIg control group.

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APPENDICES: Not applicable